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Increase in types IV and VI collagen in cherry haemangiomas*

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Summary. The capillaries in cherry haemangiomas show perivascular hyalinized sheaths. In order to clarify the nature of this sheath material, the extracellular matrix of cherry haemangiomas from 20 normal volunteers (age range 30–64 years) was investigated using immunohistochemical and electronmicroscopical methods. Antibodies against collagen types III, IV and VI and laminin were used. Hyaluronic acid was visualized using the hyaluronic acid binding region of the cartilage proteoglycan as ligand. Electronmicroscopically, the sheaths contained multilaminated basement membrane-like material, collagen fibres 20–25 nm thick with a periodicity of 67 nm and broad-banded aggregates with a periodicity of 100 nm (zebra bodies or fibrous long-spacing fibres). Immunohistochemically, type IV collagen was stained throughout the whole sheath material. Staining for laminin was more confined to the endothelial side of the sheath. Intense staining for type III collagen and hyaluronic acid was found in the connective tissue of the subpapillary layer and between the cherry haemangioma capillaries. Much weaker staining for type III collagen and no staining for hyaluronic acid were found invariably in an area 4–10 µm thick directly around the capillaries. Both sheath material and intercapillary connective tissue of the haemangiomas showed pronounced staining for collagen type VI. Immunogold staining revealed that type VI collagen was localized to microfibrils 5–6 nm thick and to the broad-banded aggregates with 100 nm periodicity. These findings further underline the assumption that the broad-banded aggregates consist of type VI collagen.

Key words: Cherry haemangioma – Type IV collagen – Type VI collagen – Immunohistochemistry – Ultrastructure

Cherry haemangiomas (senile angiomas [41] or Campell de Morgan's spots [33]) are benign tumours of subpapillary capillaries. They mainly derive from the venous limb of the capillary loops and are arranged lobular-like [41, 2, 23]. The capillaries in mature cherry haemangiomas are often surrounded by a perivascular sheath of extracellular material. This sheath is as thick as 10 µm and stains eosinophilic and PAS positive [41]. It has been suggested that the sheath material comprises 'degenerated' or 'homogenized" collagen [23, 41], but its nature and origin are still unclear. Electronmicroscopically, multilaminated basement membrane-like material [2, 43], thin collagen or 'reticulin' fibres [2, 43] and zebra bodies or fibrous long-spacing fibres [2] have been observed around cherry haemangioma capillaries.

To further clarify the nature of the sheath material, we analysed the distribution of different extracellular matrix components in cherry haemangiomas with immunohistochemical methods. To identify basement membrane components in the sheath material we used antibodies against the typical basement membrane proteins, type IV collagen and laminin [11, 31]. We also stained type III collagen, which is regarded as the major component of reticulin fibres in the adventitial dermis [29]. In previous ultraimmunohistochemical studies we were able to demonstrate that in the human eye, broad-banded aggregates similar to zebra bodies or fibrous long-spacing fibres stain with polyclonal antibodies against type VI collagen [25, 37]. We therefore studied the localization of type VI collagen in the sheath material of cherry haemangioma capillaries both light- and electron-microscopically.

Materials and methods

Cherry haemangiomas

Cherry haemangiomas from 20 normal healthy volunteers (30–64 years of age, mean 47.8 years) were investigated. The haemangiomas measured 0.5–3 mm in diameter and were removed from the skin of the trunk. One haemangioma was obtained from the upper arm. Three of the volunteers contributed multiple haemangiomas. An intradermal ring of anaesthesia was produced around the haem-

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angioma with 1% lignocaine without epinephrine. Discs of skin as thick as 3–4 mm containing the haemangioma were excised with a skin trephine from the centre of the ring and processed both for light and electron-microscopy.

Light microscopy

For paraffin sections, five samples were fixed in 10% phosphate-buffered formalin for 2 days and six samples in phosphate-buffered periodate–lysine paraformaldehyde (PLP) solution [32] for 4 h at 4 °C. The samples came from different donors. For demonstration of hyaluronic acid, three samples from different donors were fixed in 4% buffered formaldehyde containing 1% cetyl pyridium chloride for 4 h. After washing in phosphate-buffered saline (PBS), the material was embedded in paraffin. Sections were cut 5 µm thick and were either stained by Crossmon's trichrome stain [6] or further processed for immunohistochemistry.

For frozen sections, three samples were quick-frozen in isopentane, precooled with liquid nitrogen. Sections 10 µm thick were fixed in acetone for 10 min at −20 °C.

Electron-microscopy

Eight haemangiomas were fixed in 2.5% phosphate-buffered glutaraldehyde for at least 24 h at 4 °C. After postfixation with 2% osmium tetroxide, the samples were embedded in Araldit (Roth, Karlsruhe, FRG). Semithin sections were stained with toluidine blue. Ultrathin sections were contrasted with lead citrate and uranyl acetate and viewed with a Zeiss EM 902 electron microscope.

Immunohistochemistry

After fixation, the frozen sections were preincubated for 45 min in Blotto's dry milk solution [7]. The paraffin sections were deparaffinized and preincubated first with 0.1% bovine testicular hyaluronidase (Serva, Heidelberg, FRG) for 20 min, followed by 0.1% pronase E from *Streptomyces griseus* for 20 min and Blotto's solution for 30 min.

Laminin and types IV and VI collagen. After preincubation, the sections were treated with the primary antibody for 90 min. For demonstration of type IV collagen, monoclonal mouse antibodies from Heyl, Berlin, FRG (anti-human IgM, Kappa) and from Dakopatts, Hamburg, FRG (anti-human, IgG1 Kappa, CIV22 [34]) were used at a dilution of 1 : 100. For laminin, polyclonal rabbit anti-human antibodies from Heyl [1] were applied to the slides at a dilution of 1 : 10. Type VI collagen was demonstrated using a monoclonal mouse antibody (anti-human, Ig1, Kappa) and polyclonal rabbit anti-human antibodies (both from Heyl [42]) at a dilution of 1 : 50. After washing in PBS, the sections were incubated with fluorescein-labelled rabbit anti-mouse IgG (Dakopatts) or sheep anti-rabbit IgG diluted in PBS (1 : 20). The sections were mounted in Entellan (Merck, Darmstadt, FRG) containing 2.5% 1,4-diazobicyclo-octane (Merck [19]) and viewed with a Leitz Aristoplan microscope (Ernst Leitz GmbH, Wetzlar, FRG). A Kodak T-max 400 film was used for photography.

Type III collagen. The slides were incubated in 1% H_2O_2 for 10 min and in 10% bovine serum albumin (Sigma) for 20 min. Mouse monoclonal antibodies against human type III collagen from Heyl (IgG2a, Kappa) were applied to the slides at a dilution of 1 : 50 for 60 min, followed by biotinylated antibodies against mouse Ig (Amersham, Braunschweig, FRG), raised in sheep, diluted 1 : 200 for 30 min. Then the sections were incubated with a streptAB-Complex (Amersham) for 45 min. Finally, diaminobenzidine (Sigma) staining for peroxidase activity was performed as described by Graham and Karnovsky [13].

Hyaluronic acid. Staining for hyaluronic acid was performed as described previously [26]. In brief, the hyaluronic acid-binding region of the cartilage proteoglycan was isolated, linked with biotin and used in histological sections as a ligand for hyaluronic acid. Staining was performed using the avidin–biotin–perioxidase technique.

Immunoelectron-microscopy

Samples (three from different donors) were fixed in PLP for 4 h. After fixation, the samples were infiltrated with London Resin (LR) white (London Resin Co., Basingstoke, UK). Ultrathin sections were mounted on nickel grids. A drop of Blotto's dry milk solution was applied to the grids for 20 min at room temperature. After washing in PBS, the grids were incubated overnight at 4 °C with the polyclonal antibodies against type VI collagen (1 : 50). After ten 3-min changes in PBS, the grids were preincubated in normal goat serum (Sigma) for 30 min at room temperature. After washing three times in PBS, the grids were incubated for 30 min in a 1 : 20 dilution of goat anti-rabbit immunogold (10 nm, Sigma). Unbound gold was removed by ten 3-min changes in PBS. The grids were fixed with 2% glutaraldehyde for 5 min, washed and contrasted with uranyl acetate and lead citrate before examination with the electron microscope.

Controls. Control experiments were performed using either PBS or a mouse or rabbit pre-immune serum (Sigma) instead of the primary antibody. Control sections for hyaluronic acid staining were incubated for 120 min at 37 °C with 50 U/ml of *Streptomyces* hyaluronidase in the presence of various protease inhibitors (Seikagaku Fine Biochemicals, Tokyo, Japan) [26].

All antibodies had been tested using ELISA, immunoblot and immunohistology/assays.

Results

Light microscopy

The cherry haemangiomas investigated in this study showed the typical histological characteristics described

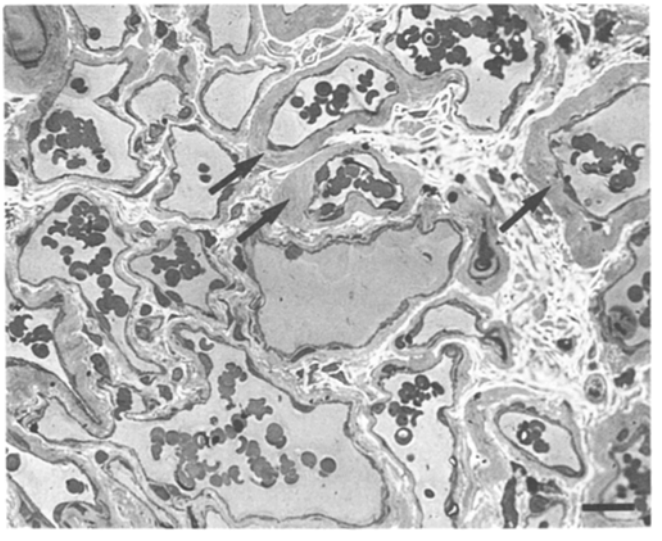


Fig. 1. Semithin section of a cherry haemangioma from a 32-year-old donor (toluidine blue; × 300; *bar* 23 µm). The capillaries show dilated lumina lined with flattened endothelial cells and are surrounded by a homogeneous sheath of extracellular material *(arrows)*, which is often as thick as 10 µm

previously [2, 23, 41, 43]. In small lesions, most of the capillaries were narrow and lined by prominent endothelial cells. Some of the capillaries, however, showed diluted lumina lined with flattened endothelial cells. These capillaries were surrounded by a homogeneous sheath of extracellular material which was often as thick as 10 μm (Fig. 1). Such capillaries predominated in the mature, larger cherry haemangiomas. The morphology of the extracellular material between the capillaries and their sheaths did not obviously differ from that of the subpapillary region of normal skin from corresponding regions.

Electron microscopy

The endothelial cells of cherry haemangioma capillaries were surrounded by an incomplete layer of pericytes and a continuous basal lamina. A characteristic feature in most of the capillaries was the additional formation of multiple layers of basal lamina-like material which surrounded the capillaries concentrically. The number of these layers differed between individual capillaries. In the narrow capillaries with prominent endothelial cells, there were only 2–3 additional layers present. Between the layers, electron-lucent ground substance with interspersed microfibrils 5–10 nm thick and collagen fibrils 20–25 nm thick with the typical periodicity of 67 nm were usually observed. In contrast to this, the number of additional layers increased considerably in the dilated capillaries with flattened endothelial cells, and thus covered a perivascular area as thick as 5 μm (Fig. 2 A, B). Around those capillaries in which light microscopy had shown the presence of the homogeneous sheath material, numerous layers of the basal lamina-like material were invariably observed. Here, the layers were embedded in moderate electron-dense flocculent or granular material (Fig. 2). In addition, there were numerous microfibrils 5–10 nm thick present. The microfibrils were aligned with numerous broad-banded aggregates resembling zebra collagen or long-spacing collagen (Fig. 2). The aggregates consisted of alternating light- and dark bands spaced with a periodicity of 100 nm (Fig. 2C). In general, these

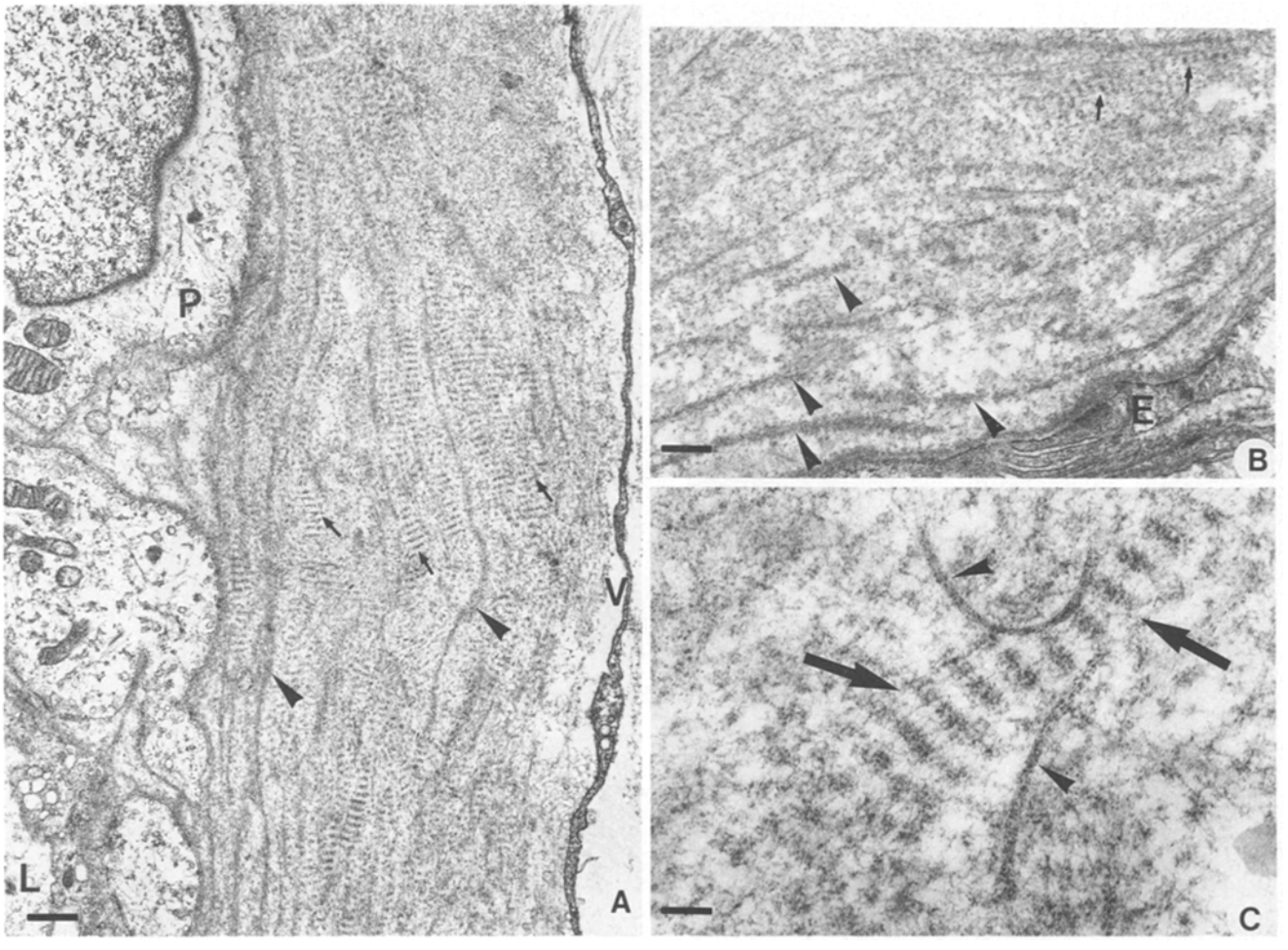
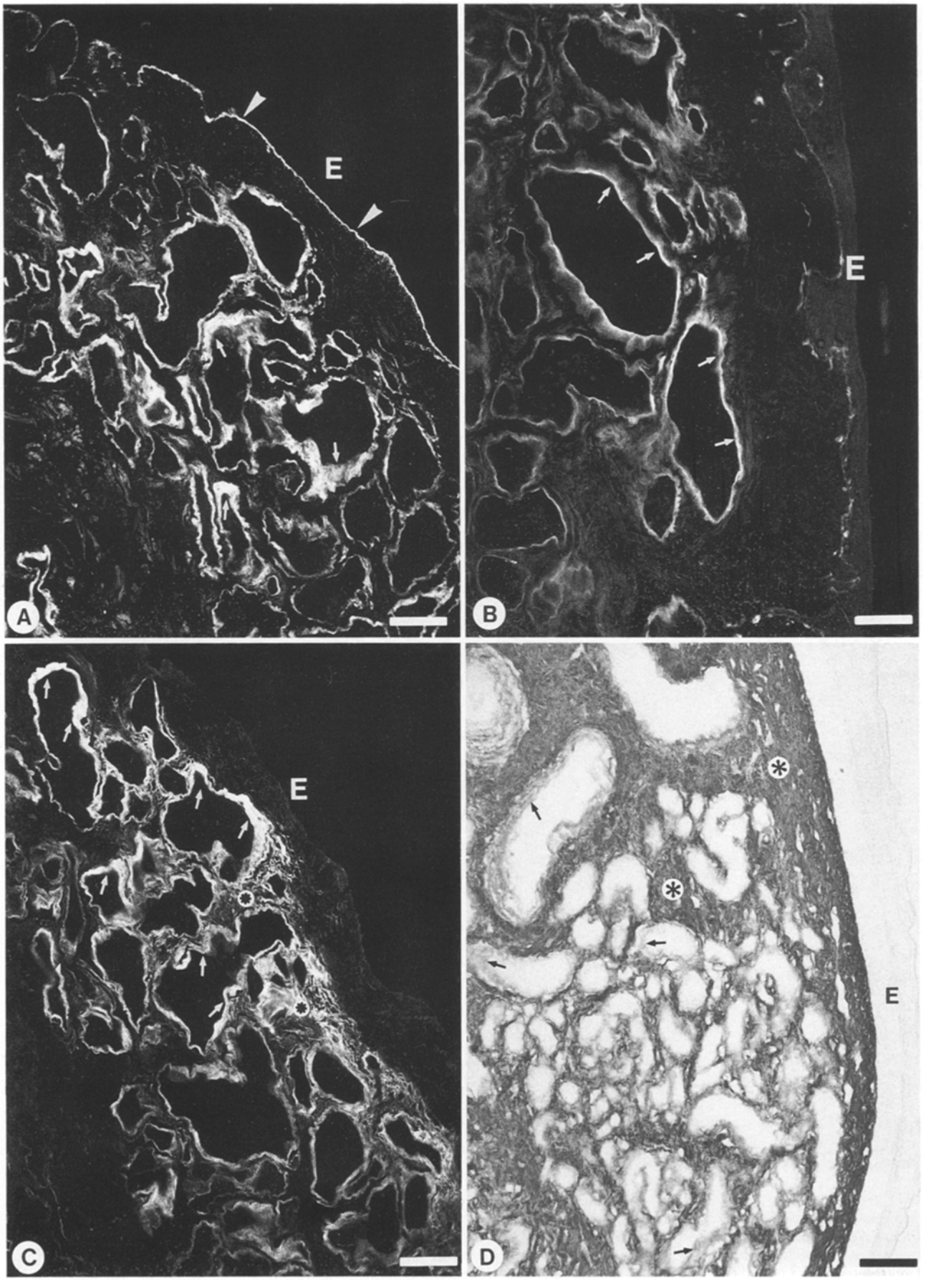


Fig. 2 A–C. Electronmicrographs of the cherry haemangioma sheath material, same case as in Fig. 1. **A, B** Multiple layers of basal lamina-like material *(arrowheads)* are embedded in moderate electron-dense granular material. In addition, numerous broad-banded aggregates with a periodicity of 100 nm are seen *(arrows)*. The sheath material is separated from the intercapillary stroma by veil cells (*V*). L, capillary lumen; *P*, pericyt; *E*, endothelial cell. (**A** × 12600, *bar* 0.56 μm; **B** × 15600, *bar* 0.45 μm). **C** Higher magnification of **A** (× 60000, *bar* 117 nm). The broad-banded aggregates are made up of alternating light and dark bands, spaced with a periodicity of 100 nm *(arrows)*. In addition, scattered collagen fibrils 20–25 nm thick *(arrowheads)* are seen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banded structures were in intimate association with the profiles of the basal lamina-like material. Native collagen fibrils with typical periodicity were only occasionally observed in these regions (Fig. 2C). In contrast to this, in the intercapillary stroma outside the sheath material large numbers of collagen fibrils were seen. Here, the ground substance stained electron-lucent and basal lamina-like material and broad-banded aggregates were not present. The pericapillary sheath material was often separated from the intercapillary stroma by fibroblasts showing characteristically long cytoplasmic processes (veil cells [46]). In many capillaries, the processes of the veil cells seemed to completely surround the sheath material (Fig. 2A).

Immunohistochemistry

Type IV collagen. The subpapillary region of normal skin showed a thin line of positive staining around nerves and capillaries and a broader line underneath the epidermis. Thus nerval, endothelial and epidermal basal lamina were stained selectively. In cherry haemangiomas, type IV collagen was stained in multilayered circular arrays around most of the capillaries (Fig. 3A). Around capillaries in which conventional histological stains indicated the presence of the typical sheath material, the whole sheath showed homogeneous positive staining for type IV collagen (Fig. 3A).

Laminin. In general, the staining for laminin corresponded to the staining for type IV collagen. Only in capillaries where thick sheath material was observed was staining for laminin not homogeneous being found only on the endothelial side of the sheath (Fig. 3B).

Type VI collagen. Pronounced positive staining for type VI collagen was seen around all cherry haemangioma capillaries (Fig. 3C). Type VI collagen stained most intensely and evenly distributed in the sheath material. However, in contrast to the staining for type IV collagen and laminin, positive staining was also seen throughout the entire intercapillary stroma of the cherry haemangiomas. In contrast to this, in normal skin from comparable regions much weaker staining for type VI collagen was seen. Only around vessels and nerves and immediately adjacent to the basal lamina of the epidermis was weak staining observed.

Type III collagen. The collagenous fibres throughout the whole subpapillary layer stained positively for type III collagen. The staining was, however, much weaker in an area 4–10 μm thick around the individual cherry haemangioma capillaries (Fig. 3D).

Hyaluronic acid. Hyaluronic acid was positively stained in the same regions where positive staining for type III collagen was also observed. An area 4–10 μm thick around the cherry haemangioma capillaries remained unstained (Fig. 4).

Fig. 3 A–D. Immunohistochemistry of a cherry haemangioma of a 40-year-old (**A–C**) and 57-year-old (**D**) donor. **A** Staining for type IV collagen. The sheath material of the capillaries stains intensely for type IV collagen *(arrows)*. In addition the basal lamina of the epidermis (*E*) is stained *(arrowheads)*. **B** Staining for laminin is confined to the endothelial side of the sheath material *(arrows)*. **C** Type VI collagen stains intensely and evenly distributed in the sheath material *(arrows)*. Positive staining is also seen throughout the entire intercapillary stroma of the haemangioma *(asterisks)*. **D** Staining for type III collagen. The collagenous fibres throughout the whole subpapillary layer stain positively for type III collagen *(asterisks)*. The staining is much weaker in an area 4–10 μm thick around the individual cherry haemangioma capillaries *(arrows)*. *E*, epidermis (paraffin sections, ×280, *bar* 35.7 μm)

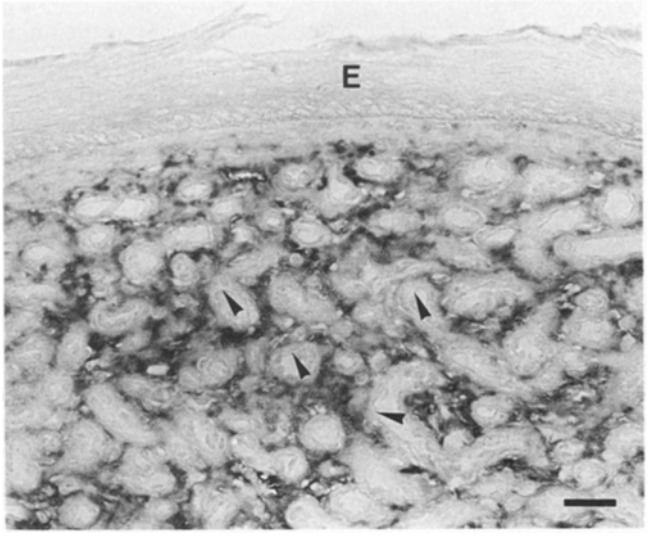

Fig. 4. Cherry haemangioma of a 31-year-old donor stained for hyaluronic acid (paraffin section, ×200, *bar* 35 μm). The intercapillary stroma of the haemangioma is positively stained, while no staining is observed in an area 4–10 μm thick around the capillaries *(arrowheads)*

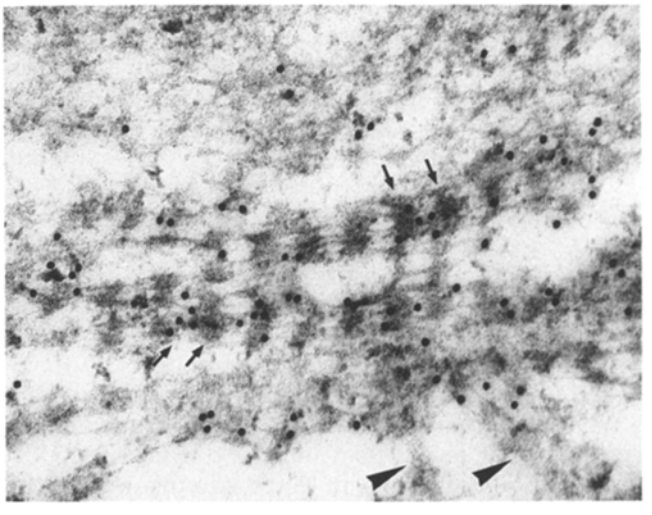

Fig. 5. Immunoelectron microscopy, using polyclonal antibodies to type VI collagen and gold-conjugated secondary antibodies (donor age, 62 years; ×98000). Gold particles are localized to microfibrils and to the broad-banded aggregates *(arrows)*. Collagen fibrils with periodicity of 67 nm are not labelled *(arrowheads)*

Ultraimmunohistochemistry

Immunoelectron microscopy, using polyclonal antibodies against type VI collagen and gold-conjugated secondary antibodies, demonstrated that type VI collagen was strictly localized to microfibrils and to the broad-banded aggregates, but not to thin collagen fibrils with a periodicity of 67 nm (Fig. 5). Gold particles labelled both the longitudinal, filamentous transverse interbands and the dark bands of the broad-banded aggregates. No labelling was seen in control sections.

Discussion

The capillaries of cherry haemangiomas showed similar ultrastructural characteristics, such as dilated venous capillaries of postcapillary venules, to normal human skin [2, 43]. A distinctive common feature of all these vessels was the presence of a multilaminated basal lamina [2, 43, 46]. In addition, however, we found a pronounced thickening of the vascular wall around many of the cherry haemangioma vessels, corroborating the findings of Braverman and Keh-Yen [2]. Ultrastructurally, these vessels were surrounded by a mainly homogeneous sheath of basement membrane-like material admixed with scattered collagen fibrils. Indeed, immunohistochemistry showed that type IV collagen and laminin, both essential macromolecules of basement membranes (for review see references [11, 31], are major components of this sheath material.

Staining for type IV collagen was seen throughout the whole sheath material, whereas staining for laminin was more confined to the endothelial cell side. Comparable differences in localization of type IV collagen and laminin have been observed in normal basement membranes like those of the epidermis [10], of the glomerular capillary loops of murine [28] and rat [5] kidney, and of the vessels in the human iris [37]. The reason for the increased production of type IV collagen is not clear, but factors causing abnormal angiogenesis might also stimulate the production of excessive amounts of extracellular matrix. On the other hand, in vitro experiments have shown that basement membrane components, such as type IV collagen, promote differentiation and inhibit proliferation of vascular endothelial cells (for review see reference 27). Thus in situ basement membrane material might exhibit similar capabilities. An increased production of this material in benign vascular tumours, such as cherry haemangiomas, might be important for regulation and organization of vascular proliferation.

In accordance with previous studies [9, 44, 29], type III collagen and hyaluronic acid stained intensely in the normal subpapillary connective tissue surrounding the haemangiomas and in the connective tissue matrix between the capillary loops. In contrast to this, staining for type III collagen was very weak in the thickened vascular wall of the haemangioma capillaries; in these areas there were also ultrastructurally only a few scattered collagen fibrils with a periodicity of 67 nm. Hyaluronic acid seemed not to be a major component of the thickened vascular wall in cherry haemangiomas, as no staining was found there.

Interestingly, we also observed a marked increase in type VI collagen which showed pronounced staining in the thickened vascular wall, but also throughout the perivascular connective tissue between the capillaries. In accordance with previous studies [4, 21], we observed that, in areas of normal skin surrounding the haemangiomas, there was only weak staining for type VI collagen in the subpapillary connective tissue near the epidermal–dermal junction and in a thin area around nerves and capillaries. Normally, type VI collagen seems not to be an intrinsic basement membrane component [11]. Based on immunoelectron microscopic investigations it has been suggested that, in a similar manner to most extracellular matrices, type VI collagen in the skin forms a network of filaments 6–10 nm thick independent of collagen type I/III fibrils [4, 21]. This network seems to anchor collagen type I/III fibres or the basement membranes of large interstitial structures, such as nerves and blood vessels, into surrounding connective tissue [21]. In accordance with our findings, an increase in type VI collagen has been observed in cerebral blood vessels in arterial hypertension [38], in experiments with haemodynamically stressed vessels [22], in the trabecular meshwork [25] and in the optic nerve [17] of eyes with increased ocular pressure. These findings might indicate that increased stress or strain on connective tissue matrices augments formation of type VI collagen.

Immunoelectron microscopic studies on the extracellular matrix of cultured fibroblasts and of rat tail tendons indicate that collagen type VI fibrils aggregate to form 100 nm 'beaded' filaments and fibrils [3, 4]. Based on these findings it has been suggested that there is a close relationship between type VI collagen and broad-banded aggregates in situ with a similar periodicity of 100 nm, which have also been referred to as long-spacing collagen, zebra collagen or Luse's bodies (for review see references 36 and 45]. Such aggregates have been described in many normal tissues including Descemet's membrane [18], the trabecular meshwork [12, 39], the synovial membrane [24, 35] and Meissner's corpuscles in the skin [15]. Recent immunoelectron microscopic studies on human trabeular meshwork [25] and synovial membrane in situ [35], and gel-cultured fibroblasts in vitro [40] confirm that there is indeed type VI collagen in these aggregates. These findings are supported by our study, as similar broad-banded aggregates in the sheath material of cherry haemangioma capillaries stain ultraimmunohistochemically for type VI collagen. There are no such aggregates in normal skin [16, 21] but in various skin lesions such as squamous cell carcinoma, malignant melanoma, primary or secondary amyloidosis, in inflammatory foci of a halonaevus and in lepromatous skin [8, 16]. Interestingly, both in skin [20] and synovial membrane [35] formation of these aggregates has been induced by a short-period incubation with bacterial collagenase or other matrix metalloproteinases.

Type VI collagen is characteristically refractory to proteolytic enzymes; this might be related to its extensive intramolecular disulphide bonding [36, 45]. If type VI

collagen fibrils form the broad-banded aggregates after collagenolytic removal of other fibrillar collagens, a similar process may acount for the formation of broad-banded aggregates around cherry haemangioma capillaries, as it is known that angiogenically stimulated capillary endothelial cells produce increased amounts of proteases and collagenases [14]. We hypothesize that type VI collagen serves as a scaffolding in tissues with high local concentrations of collagenolytic enzymes and that the increase in type VI collagen in cherry haemangiomas is related to this function.

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